Supporting Information

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SI Materials and Methods

Generation of Antigens. As we did not know whether antibodies against carbamylated proteins (anti-CarP) would be present in sera of rheumatoid arthritis (RA) patients, or which proteins they would recognize, we set out to study a diverse set of carbamylated proteins, to maximize the chances to detect as many of the anti-CarP reactivities. For this purpose we have used FCS (Bodinco) that was carbamylated, citrullinated, or left untreated. For generating carbamylated FCS (Ca-FCS), FCS was diluted in H_2O to 4 mg/mL and potassium cyanate (Sigma) was added to a concentration of 1 M. Following incubation at 37 °C for 12 h, the sample was extensively dialyzed against H_2O. Carbamylated fibrinogen (Ca-Fib) was generated by incubating 5 mg/mL fibrinogen (Fib) with 0.5 M potassium cyanate at 4 °C for 3 d followed by being extensively dialyzed against PBS. Citrullinated FCS (Ci-FCS) and citrullinated fibrinogen (Ci-Fib) was generated by incubation of 10 mg FCS or Fib in a volume of 1 mL containing 0.1 M Tris-HCl pH 7.6, 0.15 M $CaCl_2$, and 40 U PAD4 (Sigma) for 24 h at 37 °C. We have confirmed the presence of citrulline and homocitrulline residues using mass-spectrometry analysis. For Fib we observed, in the protein segments that we analyzed, extensive citrullination and complete carbamylation.

Detection of Anti-CarP Antibodies by ELISA. Nonmodified FCS and modified-FCS were coated at 10 μg/mL in 50 μL [diluted in pH 9.6 0.1 M carbonate-bicarbonate buffer (CB)] on Nunc Maxisorp plates (Thermo Scientific), overnight. Following washing in PBS containing 0.05% tween (Sigma) (PT), the plates were blocked by incubating 100 μL PBS/1% BSA (Sigma) for 6 h at 4 °C. Following additional washing, the wells were incubated with 50 μL serum at a 1/50 dilution in PBS/0.05% tween/1% BSA buffer (PTB) on ice overnight. All subsequent incubations are performed in PTB. As a standard, serial dilutions of a pool of positive sera were used. Human IgG or IgA was detected using rabbit anti-human IgG antibody (Dako) or rabbit anti-human IgA antibody (Dako) incubated on ice for 3.5 h. Following washing, wells were incubated on ice for 3.5 h with HRP-labeled goat anti-rabbit IgG antibody (Dako). Following the last washings HRP enzyme activity was visualized using ABTS, as described previously (1). Sera of healthy subjects (n = 305) were used as controls. We transformed the absorbance on both Ca-FCS and FCS to arbitrary units per milliliter (aU/mL) and subtracted the background signal (aU/mL) of FCS from the signal (aU/mL) of Ca-FCS as to analyze the specific anti-CarP reactivity (Fig. 2). We established the cut-off for a positive response as the mean plus two times the SD of the specific anti-CarP reactivity of the healthy controls.

ELISA for Fibrinogen. Nonmodified Fib Ci-Fib and Ca-Fib were coated at 20 μg/mL in 50 μL (diluted in pH 9.0 PBS) on Nunc Maxisorp plates overnight. Following washing in PT, the plates were blocked by incubating 200 μL pH 9.0 PBS/2% BSA for 2 h at 4 °C. Following additional washing, the wells were incubated with 50 μL serum at a 1/50 dilution in RIA buffer (10 mM Tris pH 7.6; 350 mM NaCl; 1% TritonX; 0.5% Na-deoxycholate; 0.1% SDS) (Sigma) on ice for 3 h. All subsequent incubations are performed in RIA buffer. As a standard, serial dilutions of a pool of positive sera were used. Human IgG was detected using HRP-labeled rabbit anti human IgG antibody (Dako) incubated on ice for 2 h. Following the last washings, HRP enzyme activity was visualized using ABTS. We analyzed sera of 214 RA patients and 54 healthy subjects as controls. We transformed the absorbance on Fib Ci-Fib and Ca-Fib to arbitrary units per milliliter. We established the cut-off for a positive response as the mean plus two times the SD of the specific anti-CarP reactivity of the healthy controls. These assays were repeated three times showing the same data.

F(ab′)2 Preparation. Total IgG from two anti-CarP–positive and two control sera were isolated via a HiTrap protein A HP column (GE Healthcare) following the protocol for the column provided by the manufacturer. F(ab′)2 fragments were generated from purified IgG samples using a F(ab′)2 Preparation Kit (Thermo Scientific) following the protocol provided by the manufacturer. We have verified the molecular nature of the intact IgG and the F(ab′)2 using Coomassie-stained SDS-PAGE gels. These F(ab′)2 were used in ELISA, as described above, now using either HRP-labeled rabbit anti human IgG, IgA, IgMκ, λ-antibody (antilight chain) (Dako), or HRP-labeled rabbit anti-human IgG (Dako).

Detection of ACPA by ELISA. Antibodies against citrullinated protein antigens (ACPA) were measured by the CCP2 ELISA (Immunoscan RA Mark 2; Eurodiagnostica). Samples with a value above 25 U/mL were considered positive according to the manufacturer's instructions. A small percentage of ACPA-positive RA patients may be outside the anti-CCP2 reactivity, and therefore both terms will be used to explicitly indicate what has been used in our analyses.

ACPA reactivity toward Ci-FCS was detected using ELISA plates that were coated with Ci-FCS (50 μL per well 10 μg/mL) diluted with CB in the Nunc Maxisorp plates overnight at 4 °C. The plates were washed in PT followed by blocking with 100 μL PBS/1%BSA solution at 37 °C for 1 h. Following washing, sera were incubated at a 1/50 dilution in 50 μL PTB and incubated at 37 °C for 1 h. After washing, human IgA and IgG were detected as described above.

Detection of Anti-CarP Antibodies by Western Blot. FCS, Ca-FCS, and Ci-FCS were loaded onto 10% SDS-polyacrylamide gels and transferred onto Hybond-C Extra membranes (Amersham). Blots were incubated in blocking buffer (3% ELK Milk/PBS/0.05% Tween) for 1 h at room temperature, following washing with PT. The blots were subsequently incubated with 2.5 mL 1:500 diluted serum in blocking buffer for 1.5 h at room temperature. The sera were either ACPA-positive anti-CarP–negative or ACPA-negative anti-CarP–positive as determined by ELISA. After three washes with PT, blots were incubated with 5 mL HRP-labeled rabbit anti-human IgG diluted in blocking buffer for 1 h at room temperature. Next, blots were washed and bound antibodies were visualized using enhanced chemiluminescence (Amersham).

Statistics of Radiological Progression. Association between anti-CarP antibodies positivity and radiographic progression was analyzed using the Statistical Package for the Social Sciences 17.0 as described earlier. P values below 0.05 were considered statistically significant. A multivariate normal regression analysis for longitudinal data were used with radiological score as response variable. This method analyses repeated measurements at once and takes advantage of the correlation between these measurements, which results in a more precise SE. Radiological scores were log-transformed to obtain a normal distribution. The rate of joint destruction over time was tested by an interaction of time with anti-CarP. The effect of time was assumed to be linear in

the interaction term. The effect of time was entered as a factor in the model as well, allowing a mean response profile over time. Age, sex, and inclusion period as proxy for treatment were included as correction variables in all analyses. In a separate analysis, the effect of anti-CarP antibodies was corrected for the effect of anti-CCP and rheumatoid factor.

1. Suwannalai P, et al. (2011) Anti-citrullinated protein antibodies have a low avidity compared with antibodies against recall antigens. *Ann Rheum Dis* 70:373–379.

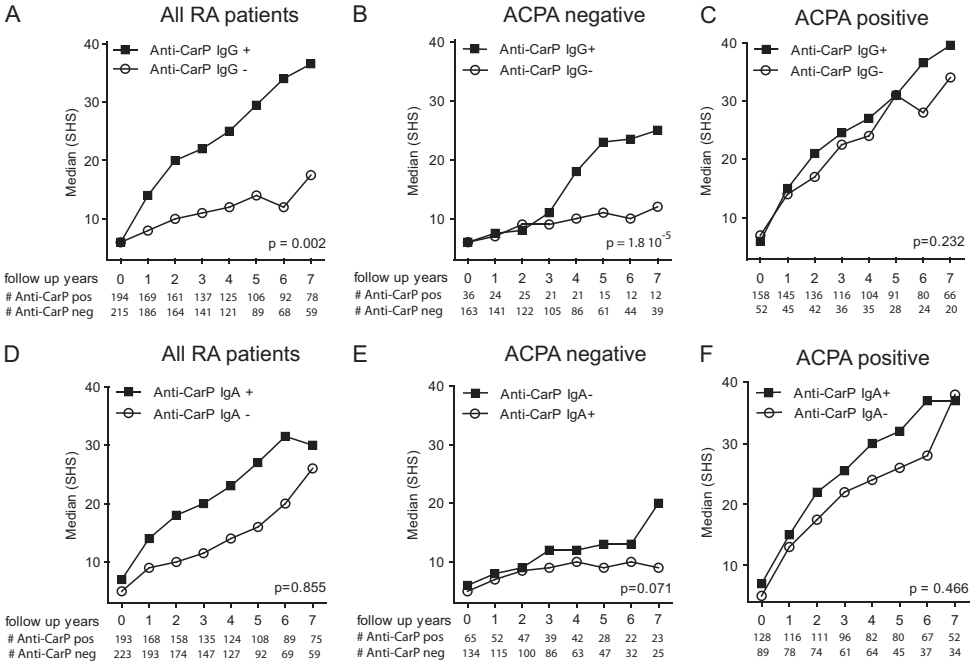

Fig. S1. Anti-CarP IgG antibodies are associated with a more severe radiological progression in RA. The extent and rate of joint destruction were analyzed in all RA patients included, or analyzed separately for ACPA-negative or ACPA-positive subgroups. The severity of joint destruction of anti-CarP IgG-positive versus -negative patients is depicted as median Sharp-van der Heijde score (SHS) on the *y* axis and the follow-up years on the *x* axis. Below the *x* axis, the patient number is listed for each time point. (*A*) Radiological progression for all RA patients analyzed, or for the (*B*) anti-CCP2–negative, or (*C*) anti-CCP2–positive patients only. Similarly, the effect of anti-CarP IgA antibodies on severity is depicted for (*D*) the RA group as a whole, or (*E*) for anti-CCP2–negative patients only, or (*F*) anti-CCP2–positive patients only. The *P* values indicated in the figure are derived from the analysis model following corrections, as described in *Materials and Methods* and *Results*.